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Phenotypic Extended-spectrum Beta-lactamase Producing *Escherichia coli* among Chickens, Cage Swabs and Wastewaters from Poultry in Indonesia

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ABSTRACT

Background: The aim of the research is to detect Extended-Spectrum Beta-Lactamase (ESBL) producing *Escherichia coli* among chickens, cage swabs and wastewaters from poultry in Indonesia.

Methods: A total of 426 samples were collected, consisting of 200 chicken small intestine contents, 149 cage swabs and 77 wastewater. Isolation and identification of *Escherichia coli* were carried out on samples, followed by antimicrobial resistance testing using the Kirby-bauer diffusion test method. Phenotypic ESBL confirmation testing uses the double disc synergy test (DDST).

Result: Of the 264 *Escherichia coli* isolates, 19.3% (n = 51) were confirmed ESBL producers, out of which 58.8% (n = 30) were obtained from ayam and 41.2% (n = 21) from poultry environments. Out of a total of 21 *Escherichia coli* isolates from poutry environments, 80.9% (n = 17) were from cage swabs and 19.1% (n = 4) from wastewaters. Based on these results, it can be seen that chickens can be considered as a transmission medium for ESBL-producing *Escherichia coli* and play a role in its spread into the environment which can ultimately pose a risk to public health. Coordinated and appropriate action is needed to reduce its impact now and in the future.

Key words: Chicken, Environment, ESBL, *Escherichia coli,* Human health.

INTRODUCTION

Escherichia coli is a commensal microorganism of the gut microbiota of healthy humans and animals, as well as an important opportunistic pathogen, which may be involved in many types of infections (Brower *et al.,* 2017; Debbarma *et al.,* 2019). The plasticity of its genome led to the evolution of this organism and made it a pathogenic strain that can cause important diseases and public health syndromes in humans and animals (Fortuna, 2022; Li *et al.,* 2020). Pathogenic *Escherichia coli* is divided into two groups depending on the location of the disease: extraintestinal pathogenic *Escherichia coli* (ExPEC) and intestinal pathogenic *Escherichia coli* (InPEC) (Pokharel *et al.,* 2023). ExPEC strains are associated primarily with neonatal meningitis and urinary tract infections in adults (Poolman and Wacker, 2016). InPEC strains are associated with diarrheal disease (Rojas-Lopez *et al.,* 2018). ExPEC is the etiological agent of colibacillosis in chickens, which is zoonotic and causes various infections in humans (Mellata, 2013). Studies show that poultry, including chickens, can be a source of transmission of *Escherichia coli* to humans (Stromberg *et al.,* 2017). Human diseases associated with *Escherichia coli* pose a large economic burden due to medical costs and lost productivity (Mitchell *et al.,* 2015).

The most frequent cause of both community-acquired and hospital-acquired extraintestinal infections, such as bloodstream, kidney and urinary tract infections (UTI), is extraintestinal pathogenic *Escherichia coli* (ExPEC). In the United States, extraintestinal infections are thought to cause more than 7 million doctor visits, 1 million ER visits and 100,000 hospitalizations annually. According to estimates, the annual expenses of these illnesses in the US range

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from $1 billion to $1.6 billion (including indirect expenditures) (Manges, 2016). ExPEC infections are a significant source of financial losses for the poultry industry and are crucial to human health. Infections with ExPEC in humans and birds cost the US economy more than $4 billion annually (Mellata, 2013; Stromberg *et al.,* 2017). Health workers and poultry workers should be educated that people around poultry are a high-risk group for fecal transmission *of Escherichia coli* and should pay attention to biosecurity and good management practices on their farms (Aworh *et al.,* 2021).

Antibiotics are widely used medically in disease prevention (*e.g.*, prophylaxis and metaphylaxis), treatment and growth promotion (Manyi-Loh *et al.,* 2018). It is estimated that two-thirds of global antimicrobials are consumed in the livestock sector (Hosain *et al.,* 2021). Based on a report on antimicrobial agents intended for use in animals published in 2021, 26% of 160 countries analyzed in 2019 still used antibiotics as growth promoters in animal production (Lekagul *et al.,* 2023). Several studies show that the widespread use of antibiotics in poultry farming contributes to an increase in antimicrobial resistance (AMR) (Hedman *et al.,* 2020).

The beta-lactamase enzyme is the biggest cause of antimicrobial resistance, especially in *Escherichia coli* (Wardhana *et al.,* 2021). Dierikx *et al.* (2013) stated that *Escherichia coli* from chicken farms has experienced a high prevalence of resistance to several antibiotics because it can produce Extended Spectrum Beta Lactamase (ESBL). ESBL is an enzyme that can hydrolyze penicillin, cephalosporin and monobactam antibiotics and causes resistance to all of these antibiotics (Akpaka *et al.,* 2021). ESBL-producing *Escherichia coli* which causes multidrug resistance (MDR) often causes infections that are difficult to treat and can be transmitted quickly through food of animal origin so that the impact on health is even wider (Brendecke *et al.,* 2022). Currently, ESBL-producing bacterial infections have become widespread and are often found in humans (Yousefipour *et al.,* 2019). The extensive use of antimicrobials in poultry farming exposes humans to antimicrobial-resistant bacteria through direct and indirect pathways (Hedman *et al.,* 2020). These include exposure through direct contact with livestock or contaminated food products, indirect gene transfer across bacterial species and widespread release of antimicrobial-resistant pathogens into the environment (Brower *et al.,* 2017). Chickens with *Escherichia coli* in their bodies have the potential to spread resistance genes in the environment which can affect human health (Aworh *et al.,* 2020). A health threat will occur if the *Escherichia coli* bacteria that contaminate the environment are ESBL-producing bacteria (Islam *et al.,* 2023). A report conducted by Larsson and Flach (2022) shows that traces of antibiotics, bacteria that are resistant to these antibiotics and genes that cause resistance in bacteria can spread in the environment.

The relationship between livestock and the emergence of ESBL in humans and the transmission of ESBL-producing bacteria must receive special attention, plus the literature regarding the spread of ESBL-producing organisms in Indonesia is still very limited (Widodo *et al.,* 2020). Based on the description above, it is necessary to carry out research regarding the prevalence of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* among chickens, cage swabs and wastewaters from poultry in Indonesia, which can then be used in determining efforts to control, spread and prevent epidemics of producing bacteria. ESBL, as well as increasing public awareness.

MATERIALS AND METHODS

Ethical clearance

Animal ethich approval was obtained *via* the ethical clearance committee of the Faculty of Veterinary Medicine, Universitas Wijaya Kusuma Surabaya, Indonesia (ethics no: 86-KKE/2022).

Study area and sample collection

Samples were taken from broiler chicken farms in Lekok, Grati and Rejoso Districts, Pasuruan Regency, Indonesia. Sampling was carried out from March 2023 to August 2023. Samples consisted of broiler chicken intestine contents, cage swabs and wastewater. Sampling of chicken intestinal contents was carried out by slaughtering broiler chickens, then abdominal dissection was carried out to take the contents of the small intestine. Samples were placed in sterile plastic which had been prepared to prevent microbial contamination from the environment. The coop swab sample was taken from a swab on the wall of the chicken coop using a sterile cotton swab, then the sample was put into a tube containing sterile Buffered Peptone Water (HiMedia, India) during transportation. Wastewater samples are taken from rivers or ditches closest to the farm in the amount of 50 ml and then put into sterile plastic bottles for transportation. Samples are stored in a cool box while traveling to the laboratory for research. A total of 426 samples were collected in this study, consisting of 200 chicken small intestine contents, 149 cage swabs and 77 wastewaters.

Microbiological analysis

The *Escherichia coli* enrichment stage was carried out by placing the sample in a test tube containing Buffered Peptone Water (HiMedia, India). Samples were incubated at 37°C for 24 hours. *Escherichia coli* was isolated using selective Eosin Methylene Blue Agar (EMBA) media (Oxoid, England) and incubated for 20-24 hours at 37°C. Next, purification was carried out by streaking a single colony of *Escherichia coli* with a sterile loop from the primary isolate to the new EMBA media. Identification of *Escherichia coli* is carried out using two methods, namely Gram staining and biochemical tests. The biochemical tests in this study consisted of Indol-Motility, Methyl Red, Voges Proskauer, Citrate and Triple Sugar Iron Agar (Effendi *et al.,* 2021; Wibisono *et al.,* 2021).

Antibiotic susceptibility testing

Antimicrobial susceptibility testing was by Clinical and Laboratory Standards Institute standard procedures using the Kirby-Bauer Diffusion Test method on Mueller Hinton Agar (MHA) media (HiMedia, India. The disk disc (Oxoid, England) used in this study consisted of ciprofloxacin 5 μg, nalidixic acid 30 μg, enrofloxacin 5 μg, erythromycin 15 μg, tetracycline 30 μg, streptomycin 10 μg, gentamicin 10 μg, ampicillin 10 μg, aztreonam 30 μg and amoxicillin-clavulanate 30 μg. The *Escherichia coli* isolate was added to physiological NaCl according to the equation 0.5 Mc. Farland (1.5×10^8 CFU/ml). The suspension that has been made is then spread evenly over the surface of the MHA media using a sterile swab. The antibiotic disc is placed on the surface of the MHA media that has been swabbed at a distance of 25-30 mm and incubated at 35°C±2°C for 16-18 hours under aerobic conditions. Interpretation of inhibition results by measuring the diameter of the inhibition zone formed by Clinical and Laboratory Standards Institute 2018 (Musa *et al.,* 2020).

ESBL confirmation testing using double disc synergy test (DDST)

Double disc synergy test (DDST) testing was carried out on isolates that were positive for multidrug resistance (MDR). DDST is carried out by placing a combination of cephalosporin antibiotic disks and clavulanic acid (a beta-lactamase enzyme resistance inhibitor) on the surface of MHA media in which *Escherichia coli* bacteria have been planted. DDST in this study used the antibiotic disk amoxicillin-clavulanate 30 μg, ceftazidime 30 μg, aztreonam 30 μg and cefotaxime 30 μg. MHA medium was incubated at 37°C for 24 hours. The formation of a keyhole inhibition zone or a picture of synergy between cephalosporin antibiotic disks and clavulanic acid indicates that the bacteria are positive for producing Extended Spectrum Beta Lactamase (Prayudi *et al.,* 2023).

RESULTS AND DISCUSSION

Overall, 426 samples consisting of chicken small intestine contents (n = 200), cage swabs (n = 149) and wastewater (n = 77) were collected from 40 broiler chicken farms. The overall prevalence of *Escherichia coli* from all sources was 60.6% (n = 264) out of which 58.3% (n = 154) were obtained from chickens and 41.7% (n=110) from poultry environments. Out of a total of 110, *Escherichia coli* isolates from poultry environments, 60.9% (n = 67) were from cage swabs and 39.1% (n = 43) from wastewater. The sample that has high prevalence of *Escherichia coli* was chicken small intestine contents.

The results of the antimicrobial susceptibility test against *Escherichia coli* showed that there was high resistance to enrofloxacin (87.1%), erythromycin (78.8%), ampicillin (75%), nalidixid acid (67.8%) and tetracycline (62.1%). To a lesser extent, *Escherichia coli* was resistant to streptomycin (58.7%), ciprofloxacin (42.1%) and gentamycin (41.3%). Very low resistance rates were seen with the antibiotics aztreonam (14.4%) and amoxicillin-clavulanate (7.2%) (Table 1).

Of 264 *Eserichia coli* isolates, 78.4% (n = 207) were multidrug resistant (MDR). Out of these, 60.9% (n = 126) were chicken isolates, while 39.1% (n = 81) were poultry environmental isolates. Out of a total of 81, *Escherichia coli* isolates from poultry environments, 70.4% (n = 57) were from cage swabs and 29.6% (n = 24) from wastewater.

Of the 264 *Escherichia coli* isolates, 19.3% (n = 51) were confirmed ESBL producers by phenotypic characterization (Fig 1). The prevalence of ESBL producing *Escherichia coli* from chickens was 58.8% (n = 30), while from poultry environments it was 41.2% (n = 21).

Based on the research results, seven types of multidrug resistance patterns were obtained in ESBL-producing *Escherichia coli*. The combination "FQ-AM-M-TE-BL" was the most frequent at 41.2%, followed by the combination "FQ-AM-M-BL" at 23.5%. The results showed that the majority of ESBL-producing *Escherichia coli* had multidrug resistance to 5 antimicrobial classes (AMC) as much as 41.2% (21/51), the remainder had multidrug resistance to 4 AMC as much as 39.2% (20/51) and 3 AMC as much as 19.6% (10/51), can be seen in Table 2.

The results showed that *Escherichia coli* had high resistance to enrofloxacin (87.1%), erythromycin (78.8%),

Table 1: Antimicrobial resistance profile of *Escherichia coli* isolates from chickens and poultry environments.

Class of antibiotics	Antibiotics	Resistance inhibition zone (mm)	Chickens (n = 154)	Cage swabs (n = 67)	Wastewater (n = 43)
Fluoroquinolone	Ciprofloxacin (CIP)	≤15	65 (42.2%)	34 (50.8%)	12 (27.9%)
	Enrofloxacin (ENR)	≤24	138 (89.6%)	56 (83.58%)	36 (83.7%)
	Nalidixic acid (NA)	≤13	115 (74.7%)	49 (73.1%)	15 (34.8%)
Macrolide	Erythromycin (E)	≤13	107 (69.5%)	59 (88.1%)	42 (97.7%)
Aminoglycoside	Streptomycin (S)	≤11	78 (50.6%)	45 (67.2%)	32 (74.4%)
	Gentamycin (CN)	≤12	75 (48.7%)	25 (37.3%)	9 (20.9%)
Tetracycline	Tetracycline (TE)	≤11	95 (61.7%)	48 (71.6%)	21 (48.8%)
Beta-Lactam	Ampicillin (AMP)	≤13	124 (80.5%)	57 (85.1%)	16 (37.2%)
	Aztreonam (ATM)	≤17	15 (9.7%)	19 (28.4%)	4 (9.3%)
	Amoxicillin-clavulanate (AC)	≤13	8 (5.2%)	8 (11.9%)	3 (7%)

ampicillin (75%), nalidixic acid (67.8%) and tetracycline (62.1%). These results are the same as the results of other studies, from different regions in Indonesia, which showed that *Escherichia coli* from broilers in Sukabumi had high resistance to enrofloxacin (72%), ampicillin (100%), nalidixid acid (100%), erythromycin (92%) and tetracycline (88%) (Hardiati *et al.,* 2021). The high resistance to enrofloxacin, ampicillin, erythromycin and tetracyclin is clearly related to the high use of these antibiotics in the poultry farming sector in Indonesia (Efendi *et al.,* 2022). The use of enrofloxacin is the most popular used in poultry in Indonesia at around 60%, followed in sequence by tetracycline, erythromycin and ampicillin (Grabowski *et al.,* 2022).

This research shows that there are 19.5% ESBL-producing *Escherichia coli* (30/154) in isolates originating from chickens. This incidence is slightly lower than previous research in Indonesia which stated that there were 28.75% ESBL-producing *Escherichia coli* in broilers in the Blitar area (Wibisono *et al.,* 2021). The prevalence in this study is much lower compared to the incidence in several other Asian countries, Thailand at 70.5% (Tansawai *et al.,* 2018), India 87% (Brower *et al.,* 2017), dan Philippines at 60,26% (Gundran *et al.,* 2019). The low incidence could be because in Indonesia there is a ban on the use of antibiotic growth promoters (AGP) in the livestock sector which became effective in January 2018 referring to Minister of Agriculture Regulation No. 14/2017. In the past, almost all feed factories added antibiotics as feed additives to commercial feed, this is what caused the high incidence of antibiotic resistance in chicken farms (Chattopadhyay, 2014). However, the discovery of ESBL-producing *Escherichia coli* in chickens is a cause for concern considering that it can attack humans through contaminated chicken carcasses and can spread to the environment surrounding the poultry (Da Silva *et al.,* 2023).

The phenotypic prevalence of ESBL-producing *Escherichia coli* was 19.3%. The prevalence of ESBL producing *Escherichia coli* from chickens was 58.8% (n = 30), while from poultry environments it was 41.2% (n = 21). In this study, ESBL-producing *Escherichia coli* was not only found in chickens but also in the environment around the cage (cage swabs and wastewater), this proves that there is a spread of ESBL-producing *Escherichia coli* from chickens to the surrounding environment. This incident is strengthened by the fact that ESBL-producing *Escherichia coli* from chickens has the same multidrug resistance pattern as ESBL-producing *Escherichia coli* from cage swabs and wastewater (Patricio *et al.,* 2022). The “FQ-AM-M-TE-BL” pattern is the most common in chickens as well as in cage swabs and wastewater.

The research results of Kwoji *et al.* (2019) shows that the transfer of resistance genes in *Escherichia coli* is quite high in the poultry farming environment. Poultry farming environments often have a high concentration of microorganisms, thus triggering the creation of poor environmental quality and benefiting the spread of ESBL-producing *Escherichia coli* because the ESBL coding gene in the plasmid can be transferred to other bacteria (Yanestria *et al.,* 2022; Widodo *et al.,* 2022). Antibiotic resistance bacteria (ARB) and antibiotic-resistant genes (ARG) from destroyed ARB can spread in the environment (Courti *et al.,* 2022; Tyasningsih *et al.,* 2022). Unwise useof antibiotics in animals exacerbates the spread of ARBs and ARGs. Humans and animals excrete ARBs and ARGs through urine and feces into the environment (Newton *et al.,* 2015). Potential sources of AMR in poultry production consist of air, dust, soil, feed and rodents or other animals.

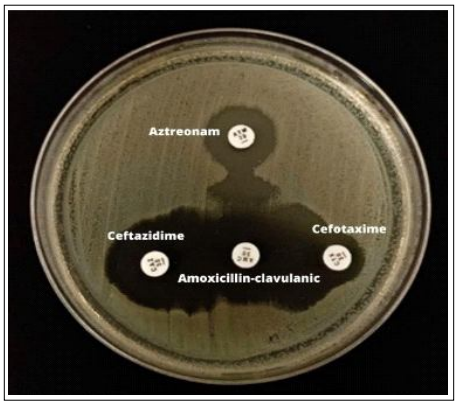


Fig 1: *Escherichia coli* was confirmed as ESBL using the DDST method.

Table 2: Data on multidrug resistance of ESBL-producing *Escherichia coli.*

Number of AMC	Multidrug resistance pattern	*Escherichia coli* (n =51)			Total
		Chicken	Cage swabs	Wastewaters	
5	FQ-AM-M-TE-BL	8	11	2	21
4	FQ-AM-M-BL	9	2	1	12
4	FQ-M-TE-BL	5	2	0	7
4	AM-M-TE-BL	0	0	1	1
3	FQ-M-BL	4	1	0	5
3	FQ-TE-BL	1	1	0	2
3	FQ-AM-BL	3	0	0	3
Total		30	17	4	51

Note: FQ: Fluoroquinolone; AM: Aminoglycosides; M: Macrolides; TE: Tetracycline; BL: Beta-lactam; AMC: Antimicrobial class.

From these sources, AMR bacteria and genes can be transmitted indirectly to the environment (Martínez-Álvarez *et al.,* 2022; Effendi *et al.,* 2018). Several studies have shown the role of air as a means of dissemination and survival both inside and outside the farm and poultry waste is considered the main vector for the spread of AMR bacteria (Vounba *et al.,* 2019; Hedman *et al.,* 2020).

Large amounts of antibiotic residues are also excreted from the animal's body (Arsène *et al.,* 2022). It is estimated that at least 70% of the total antibiotics consumed are excreted unchanged (Kümmerer, 2009). The released antibiotic, after being reduced by metabolism, has a non-killing concentration, known as a sub-inhibitory concentration (Vasilchenko and Rogozhin, 2019; Ramandinianto *et al.,* 2020). These antibiotics are mixed intensively with pathogenic bacteria in wastewater, more intense contact occurs in non-killing concentrations, the bacteria absorb the antibiotics and begin to adapt to them, eventually developing antibiotic resistance (Rizzo *et al.,* 2013; Riwu *et al.,* 2020). This statement is also reinforced by other literature from Meng *et al.* (2022) which states that waste from animals given antibiotics contains antibiotic residues and resistant bacteria. Other literature states that horizontal plasmid-mediated transfer of Antibiotic Resistance Genes (ARGs) is recognized as the most dominant way of spreading ARGs in humans, animals and the environment. Antibiotic selective pressure is considered to be one of the important contributors to promoting the spread of antibiotic resistance through horizontal gene transfer (HGT) (Liu *et al.,* 2020). ESBLs can also be spread *via* HGT, as suggested by the study of Franz *et al.* (2015) that *Escherichia coli*-producing ESBL is at risk of spreading ARGs, especially in susceptible individuals.

Various genetic mechanisms are involved in the acquisition and spread of AMR (Aminov, 2021). The *Escherichia coli* mobilome includes a variety of mobile and mobilizable genetic elements, including plasmids, transposons, insertion sequences and integrons (intl), the latter of which is known to be involved in the spread of antibiotic resistance, especially among Gram-negative bacteria (Gillings, 2014; Pradika *et al.,* 2019; Durairajan *et al.,* 2021). Integrons are genetic structures that contain AMR genes in their variable regions (as gene cassettes) and have been detected in poultry farms in various studies (Kalantari *et al.,* 2021; Perez-Etayo *et al.,* 2018). Another thing to worry about is that the ESBL-producing *Escherichia coli* in this study is multidrug-resistant (MDR), even resistant to 5 classes of antibiotics. An isolate is defined as multidrug-resistant if it is insensitive to one or more antimicrobials in ≥ 3 different antimicrobial classes (Varga *et al.,* 2019). ESBL-producing *Escherichia coli* in livestock animals has become a public health concern in recent years (Ansharieta *et al.,* 2021). Infection in humans can cause treatment failure, prolonged illness and increase the death rate, especially in patients with septicemia and urinary tract infections (Rodroo *et al.,* 2021).

Based on the results of this research, it can be seen that poultry can be considered as a transmission medium for MDR bacteria, one of which is that ESBL plays a role in the spread of MDR into the environment which can ultimately pose a risk to public health. Antimicrobial resistance is a global and growing problem that requires a solution through a "One Health" approach (Velazquez-Meza *et al.,* 2022; Yunita *et al.,* 2020). Coordinated and appropriate action is needed to mitigate its impact now and in the future, as well as measures to ensure the economic *via*bility of the poultry sector along with public health security.

CONCLUSION

This study shows that chickens can be considered as a transmission medium for ESBL-producing *Escherichia coli* and play a role in its spread into the environment which can ultimately pose a risk to public health. It is important to increase public awareness of hygiene practices in poultry and chicken slaughtering, as well as during food processing. Control of antimicrobial use needs to be improved to reduce the spread of ESBL-producing *Escherichia coli*.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal Care and Handling Techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish or preparation of the manuscript.

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